

FORM PTO-1390		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			4121-136
			U.S. APPLICATION NO. (If known, see 37 CFR 1.5) <b>10/069056</b>
INTERNATIONAL APPLICATION NO. PCT/EP00/07835 07835	INTERNATIONAL FILING DATE 11 August 2000	PRIORITY DATE CLAIMED 13 August 1999	
TITLE OF INVENTION PARVOVIRUS NS 1 VARIANTS			
APPLICANT(S) FOR DO/EO/US NUESCH, Jurg; ROMMELAERE, Jean			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<ol style="list-style-type: none"> <li>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li> <li>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</li> <li>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> <li>a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li>6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</li> <li>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> have been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input checked="" type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).*(Unsigned)</li> <li>10. <input checked="" type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol>			
Items 11. to 16. below concern other document(s) or information included:			
<ol style="list-style-type: none"> <li>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li> <li>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</li> <li>14. <input type="checkbox"/> A substitute specification.</li> <li>15. <input checked="" type="checkbox"/> A small entity statement.</li> <li>16. <input type="checkbox"/> Other items or information:</li> </ol>			

NOTE: This application is being filed with an unsigned Oath or Declaration under the provisions of 37 CFR § 1.53 in order that applicants may secure a filing date of February 11, 2002. Upon receipt of a "Notice to File Missing Parts - Filing Date Granted," an executed Declaration and Power of Attorney, will be filed in the Patent and Trademark Office. The undersigned agent affirmatively states that she has been duly authorized and appointed to file this application on behalf of the applicants and that the Declaration and Power of Attorney to be filed hereafter will confirm the undersigned agent's authorization and appointment. Applicants are entitled to small entity status within the meaning of 37 CFR § 1.9.

17. <input checked="" type="checkbox"/> The following fees are submitted: <b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b> Search Report has been prepared by the EPO or JPO .....\$860.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) .....\$0.00 No International preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) .....\$0.00  Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO .....\$1000.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) .....\$0.00  <div style="text-align: right;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></div>				<b>CALCULATIONS</b> <b>JC13 Rec'd PCT/PTO 11 FEB 2002</b>		<b>PTO USE ONLY</b>	
				\$ 860.00			
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$			
Claims	Number Filed	Number Extra	Rate				
Total Claims	18-20 =	0	X \$18.00	\$			
Independent Claims	2- 3 =	0	X \$80.00	\$			
Multiple dependent claim(s) (if applicable)			+ \$270.00	\$			
<b>TOTAL OF ABOVE CALCULATIONS =</b>				860.00			
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$ 430.00			
<b>SUBTOTAL =</b>				\$ 430.00			
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 Months from the earliest claimed priority date (37 CFR 1.492(f)).				\$			
<b>TOTAL NATIONAL FEE =</b>				\$ 430.00			
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				\$			
<b>TOTAL FEE ENCLOSED =</b>				\$ 430.00			
				<b>Amount to be:</b>	\$		
				<b>refunded</b>			
				<b>Charged</b>	\$		

a. ☒ A check in the amount of \$430.00 to cover the above fees is enclosed.


b. ☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \$ \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 08-3284. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not yet been met, a petition to revive (37 CFR 1.127(a) or (b)) must be filed and granted to restore the application to pending status.**

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10059056 10/069056

JC13 Rec'd PCT/PTO 1.1 FEB 2002

4121-136  
PATENT APPLICATION

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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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**In re Application of:** Jurg Nuesch, et al.

**Application No.:** New U.S. National Stage Application of  
PCT International Application No. PCT/EP00/07835

**International Filing Date:** 11 August 2000

**Priority Date Claimed:** 13 August 1999 (European Appl. No. 99 115 161.4)

**U.S. National Phase Filing Date:** Date of mailing identified below

**Title:** PARVOVIRUS NS 1 VARIANTS



23448

PATENT TRADEMARK OFFICE

**EXPRESS MAIL CERTIFICATE**

I hereby certify that I am mailing the attached documents to the Commissioner for Patents on the date specified, in an envelope addressed to the Commissioner for Patents, Box Patent Application, Washington, DC 20231, and Express Mailed under the provisions of 37 CFR 1.10.

Katrina Holland

Name of Person Mailing This Document

Signature

February 11, 2002

Date

EV037733273US

Express Mail Label Number

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**PRELIMINARY AMENDMENT**

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Commissioner for Patents  
BOX PATENT APPLICATION  
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified new national phase patent application, please amend the application, as follows:

**In the Specification**<sup>1</sup>

Please insert on page 1, between the title of the application and the first paragraph, the following new paragraph:

**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application is filed under the provisions of 35 U. S.C. §371 and claims the priority of International Patent Application No. PCT/EP00/07835 filed August 11, 2000, which in turn claims priority of European Patent Application No. 99 115 161.4 filed August 13, 1999.

On page 4 please amend paragraphs 1, 2 and 3 to read as follows:

According to the invention parvovirus NS1 variants are preferred in which the shift of equilibrium is achieved by mutation of one or several phosphorylation sites. Particularly preferred are parvovirus NS1 variants which have a mutation at one or several of the phosphorylation sites 283, 363, 394 and 463. Even more preferred are the parvovirus NS1 variants S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8) and T463A (SEQ ID NO. 10), which are indicated in Table 1 and [figure 1] figures 1.1, 1.2, 1.3 and 1.4. In S283A, a serine is exchanged by an alanine at position 283, in T363A, a threonine is exchanged by alanine at position 363, in T394A a threonine is exchanged by alanine at position 394 and in T463A a threonine is exchanged by alanine at position 463.

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<sup>1</sup> Applicants has provided a marked-up version of amended paragraphs and claims 1, 2, 3, 4, 5, 6, 7, 9, 10, 11 and 12 in Appendix A, and a clean set of all pending claims, amended to date, in Appendix B.

A further subject matter of the present invention relates to a nucleic acid, particularly a DNA, which codes for an above parvovirus NS1 variant. Such a DNA comprises preferably:

- (a) the DNA of fig. 1.1 (SEQ ID NO. 3), 1.2 (SEQ ID NO. 5), 1.3 (SEQ ID NO. 7) and 1.4 (SEQ ID NO. 9), respectively
- (b) a DNA hybridizing with the DNA from (a), said DNA comprising the mutated phosphorylation site of the DNA from (a), or
- (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.

The DNA of (a) was deposited with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) on Aug. 11 1999, i.e. fig. 1.1 as Escherichia coli pRSV-NS: S283A under DSM 12994 (SEQ ID NO. 3), fig. 1.2 as Escherichia coli pRSV-NS: T363A under DSM 12995 (SEQ ID NO. 5), fig. 1.3 as Escherichia coli pRSV-NS: T394A under DSM 12996 (SEQ ID NO: 7) and fig. 1.4 as Escherichia coli pRSV-NS: T463A under DSM 12997 (SEQ ID NO. 9).

On page 7, please amend the last full paragraph to read as follows:

Fig. 1 shows the DNA and amino acid sequences of parvovirus NS1 variants according to the invention (fig. 1.1 (SEQ ID Nos. 3 and 4), 1.2 (SEQ ID Nos. 5 and 6), 1.3 (SEQ ID Nos. 7 and 8) and 1.4 (SEQ ID Nos. 9 and 10)) as compared to parvovirus NS1 wild type (SEQ ID Nos. 1 and 2). In this connection, the mutated sites in the parvovirus NS1 variants according to the invention are labeled each.

### **In the Claims**

Please amend claims 1, 2, 3, 4, 5, 6, 7, 9, 10, 11 and 12 to read as follows:

1. A parvovirus NS1 variant protein having a shifted equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b), wherein the shifted equilibrium is selected from the group consisting of:

- the activities (a) are reduced and eliminated, respectively, and activity (b) is maintained or increased; and
  - activity (b) is reduced and eliminated, respectively, and the activities (a) are maintained or increased.
2. The parvovirus NS 1 variant protein according to claim 1, wherein one or several phosphorylation sites are mutated.
  3. The parvovirus NS 1 variant protein according to claim 2, wherein the mutations are located at an amino acid residue site selected from the group consisting of: 283, 363, 394 and/or 463.
  4. The parvovirus NS 1 variant protein according to claim 3, namely the NS 1 variants S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8), and T463A (SEQ ID NO. 10).
  5. A DNA, coding for the parvovirus NS1 variant protein according to claim 1.
  6. The DNA according to claim 5, wherein the DNA comprises a member selected from the group consisting of :
    - (a) the DNA of SEQ ID Nos 3, 5, 7 and 9, said DNA comprising a mutated phosphorylation site,
    - (b) a DNA hybridizing with the DNA from (a) under high stringency conditions, said DNA comprising the mutated phosphorylation site of the DNA from (a), or
    - (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.
  7. An expression vector, comprising the DNA according to claim 6.

9. A method of producing the parvovirus NS 1 variant protein according to claim 1, comprising the culturing of the transformant according to claim 8 under suitable conditions.
10. An antibody, directed against the parvovirus NS 1 variant protein according to claim 1.
11. A Kit comprising at least one member selected from the group consisting of:
  - (a) a parvovirus NS 1 variant protein according to claim 1,
  - (b) a DNA according to claim 5,
  - (c) an antibody according to claim 10, and
  - (d) conventional auxiliary agents, such as solvents, buffers, carriers markers and controls.
12. Use of the parvovirus NS 1 variant protein according to claim 1 as a toxin for treating tumoral diseases.

Please add claims 14-18.

14. The parvovirus NS 1 variant protein according to claim 3, wherein the NS 1 variants has a mutation at sites S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8), and T463A (SEQ ID NO. 10).
15. A DNA, coding for the parvovirus NS1 variant protein according to claim 4.
16. An expression vector, comprising the DNA according to claim 5.
17. A method of producing the parvovirus NS 1 variant protein according to claim 4, comprising the culturing of the transformant according to claim 8 under suitable conditions.

18. An antibody, directed against the parvovirus NS 1 variant protein according to claim 4.



A marked-up version of amended paragraphs and claims 1, 2, 3, 4, 5, 6, 7, 9, 10, 11 and 12 is included herewith in Appendix A and a clean copy of all pending claims is included in Appendix B.

It is requested that the examination and prosecution of this application proceed on the basis of these amended claims 1-18.

Marionne Treen

### Attorney for Applicants

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**Attorney File: 4121-136**

## APPENDIX A

### In the Specification

Please insert on page 1, between the title of the application and the first paragraph, the following new paragraph:

#### CROSS-REFERENCE TO RELATED APPLICATIONS.

This application is filed under the provisions of 35 U. S.C. §371 and claims the priority of International Patent Application No. PCT/EP00/ 07835 filed August 11, 2000, which in turn claims priority of European Patent Application No. 99 115 161.4 filed August 13, 1999.

On page 4 please amend paragraphs 1, 2 and 3 to read as follows:

According to the invention parvovirus NS1 variants are preferred in which the shift of equilibrium is achieved by mutation of one or several phosphorylation sites. Particularly preferred are parvovirus NS1 variants which have a mutation at one or several of the phosphorylation sites 283, 363, 394 and 463. Even more preferred are the parvovirus NS1 variants S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8) and T463A (SEQ ID NO. 10), which are indicated in Table 1 and [figure 1] figures 1.1, 1.2, 1.3 and 1.4. In S283A, a serine is exchanged by an alanine at position 283, in T363A, a threonine is exchanged by alanine at position 363, in T394A a threonine is exchanged by alanine at position 394 and in T463A a threonine is exchanged by alanine at position 463.

A further subject matter of the present invention relates to a nucleic acid, particularly a DNA, which codes for an above parvovirus NS1 variant. Such a DNA comprises preferably:

- (a) the DNA of fig. 1.1 (SEQ ID NO. 3), 1.2 (SEQ ID NO. 5), 1.3 (SEQ ID NO. 7) and 1.4 (SEQ ID NO. 9), respectively
- (b) a DNA hybridizing with the DNA from (a), said DNA comprising the mutated phosphorylation site of the DNA from (a), or
- (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.

The DNA of (a) was deposited with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) on Aug. 11 1999, i.e. fig. 1.1 as Escherichia coli pRSV-NS: S283A under DSM 12994 (SEQ ID NO. 3), fig. 1.2 as Escherichia coli pRSV-NS: T363A under DSM 12995 (SEQ ID NO. 5), fig. 1.3 as Escherichia coli pRSV-NS: T394A under DSM 12996 (SEQ ID NO. 7) and fig. 1.4 as Escherichia coli pRSV-NS: T463A under DSM 12997 (SEQ ID NO. 9).

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Fig. 1 shows the DNA and amino acid sequences of parvovirus NS1 variants according to the invention (fig. 1.1 (SEQ ID Nos. 3 and 4), 1.2 (SEQ ID Nos. 5 and 6), 1.3 (SEQ ID Nos. 7 and 8) and 1.4 (SEQ ID Nos. 9 and 10)) as compared to parvovirus NS1 wild type (SEQ ID Nos. 1 and 2). In this connection, the mutated sites in the parvovirus NS1 variants according to the invention are labeled each.

### **In the Claims**

Please amend claims 1, 2, 3, 4, 5, 6, 7, 9, 10, 11 and 12 to read as follows:

1. A parvovirus NS 1 variant protein having a shifted equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b), wherein the shifted equilibrium is selected from the group consisting of:
  - the activities (a) are reduced and eliminated, respectively, and activity (b) is maintained or increased; and [or]

- activity (b) is reduced and eliminated, respectively, and the activities (a) are maintained or increased.

2. The parvovirus NS 1 variant protein according to claim 1, wherein one or several phosphorylation sites are mutated.
3. The parvovirus NS 1 variant protein according to claim 2, wherein the mutations are located at an amino acid residue site [sites] selected from the group consisting of: 283, 363, 394 and/or 463.
4. The parvovirus NS 1 variant protein according to claim [2 or ]3, namely the NS 1 variants S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8), and T463A (SEQ ID NO. 10).
5. A DNA, coding for the parvovirus NS1 variant protein according to claim 1. [any one of claims 1 to 4.]
6. The DNA according to claim 5, wherein the DNA comprises a member selected from the group consisting of :
  - (a) the DNA of [figure 1] SEQ ID Nos 3, 5, 7 and 9, said DNA comprising a mutated phosphorylation site,
  - (b) a DNA hybridizing with the DNA from (a) under high stringency conditions, said DNA comprising the mutated phosphorylation site of the DNA from (a), or
  - (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.
7. An expression vector, comprising the DNA according to claim [5 or] 6.
9. A method of producing the parvovirus NS 1 variant protein according to claim 1 [any one of claims 1 to 4], comprising the culturing of the transformant according to claim 8 under suitable conditions.

10. An antibody, directed against the parvovirus NS 1 variant protein according to claim 1 [any one of claims 1 to 4].
11. A Kit comprising at least one member selected from the group consisting of:
  - (a) a parvovirus NS 1 variant protein according to claim 1[the invention],
  - (b) a DNA according to claim 5 [the invention, e.g. an expression vector, particularly a parvovirus],
  - (c) an antibody according to claim 10, [the invention, as well as ] and
  - (d) conventional auxiliary agents, such as solvents, buffers, carriers markers and controls[,[wherein of components (a) to (d) one or more representatives can be present each].
12. Use of the parvovirus NS 1 variant protein according to claim 1 [one of claims 1 to 4] as a toxin for treating tumoral diseases.

## APPENDIX B

### In the Specification

Please insert on page 1, between the title of the application and the first paragraph, the following new paragraph:

#### CROSS-REFERENCE TO RELATED APPLICATIONS

This application is filed under the provisions of 35 U. S.C. §371 and claims the priority of International Patent Application No. PCT/EP00/07835 filed August 11, 2000, which in turn claims priority of European Patent Application No. 99 115 161.4 filed August 13, 1999.

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According to the invention parvovirus NS1 variants are preferred in which the shift of equilibrium is achieved by mutation of one or several phosphorylation sites. Particularly preferred are parvovirus NS1 variants which have a mutation at one or several of the phosphorylation sites 283, 363, 394 and 463. Even more preferred are the parvovirus NS1 variants S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8) and T463A (SEQ ID NO. 10), which are indicated in Table 1 and figures 1.1, 1.2, 1.3 and 1.4. In S283A, a serine is exchanged by an alanine at position 283, in T363A, a threonine is exchanged by alanine at position 363, in T394A a threonine is exchanged by alanine at position 394 and in T463A a threonine is exchanged by alanine at position 463.

A further subject matter of the present invention relates to a nucleic acid, particularly a DNA, which codes for an above parvovirus NS1 variant. Such a DNA comprises preferably:

- (a) the DNA of fig. 1.1 (SEQ ID NO. 3), 1.2 (SEQ ID NO. 5), 1.3 (SEQ ID NO. 7) and 1.4 (SEQ ID NO. 9), respectively
- (b) a DNA hybridizing with the DNA from (a), said DNA comprising the mutated phosphorylation site of the DNA from (a), or
- (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.

The DNA of (a) was deposited with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) on Aug. 11 1999, i.e. fig. 1.1 as *Escherichia coli* pRSV-NS: S283A under DSM 12994 (SEQ ID NO. 3), fig. 1.2 as *Escherichia coli* pRSV-NS: T363A under DSM 12995 (SEQ ID NO. 5), fig. 1.3 as *Escherichia coli* pRSV-NS: T394A under DSM 12996 (SEQ ID NO. 7) and fig. 1.4 as *Escherichia coli* pRSV-NS: T463A under DSM 12997 (SEQ ID NO. 9).

On page 7, please amend the last full paragraph to read as follows:

Fig. 1 shows the DNA and amino acid sequences of parvovirus NS1 variants according to the invention (fig. 1.1 (SEQ ID Nos. 3 and 4), 1.2 (SEQ ID Nos. 5 and 6), 1.3 (SEQ ID Nos. 7 and 8) and 1.4 (SEQ ID Nos. 9 and 10)) as compared to parvovirus NS1 wild type (SEQ ID Nos. 1 and 2). In this connection, the mutated sites in the parvovirus NS1 variants according to the invention are labeled each.

### **In the Claims**

- I. A parvovirus NS1 variant protein having a shifted equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b), wherein the shifted equilibrium is selected from the group consisting of:
  - the activities (a) are reduced and eliminated, respectively, and activity (b) is maintained or increased; and
  - activity (b) is reduced and eliminated, respectively, and the activities (a) are maintained or increased.

2. The parvovirus NS 1 variant protein according to claim 1, wherein one or several phosphorylation sites are mutated.
3. The parvovirus NS 1 variant protein according to claim 2, wherein the mutations are located at an amino acid residue site selected from the group consisting of: 283, 363, 394 and/or 463.
4. The parvovirus NS 1 variant protein according to claim 3, namely the NS 1 variants S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8), and T463A (SEQ ID NO. 10).
5. A DNA, coding for the parvovirus NS1 variant protein according to claim 1.
6. The DNA according to claim 5, wherein the DNA comprises a member selected from the group consisting of :
  - (a) the DNA of SEQ ID Nos 3, 5, 7 and 9, said DNA comprising a mutated phosphorylation site,
  - (b) a DNA hybridizing with the DNA from (a) under high stringency conditions, said DNA comprising the mutated phosphorylation site of the DNA from (a), or
  - (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.
7. An expression vector, comprising the DNA according to claim 6.
8. A transformant, containing the expression vector according to claim 7.
9. A method of producing the parvovirus NS 1 variant protein according to claim 1, comprising the culturing of the transformant according to claim 8 under suitable conditions.



10. An antibody, directed against the parvovirus NS 1 variant protein according to claim 4.
11. A Kit comprising at least one member selected from the group consisting of:
  - (a) a parvovirus NS 1 variant protein according to claim 4,
  - (b) a DNA according to claim 5,
  - (c) an antibody according to claim 10, and
  - (d) conventional auxiliary agents, such as solvents, buffers, carriers markers and controls.
12. Use of the parvovirus NS 1 variant protein according to claim 1 as a toxin for treating tumoral diseases.
13. Use of the DNA according to claim 7 as a vector for gene therapy.
14. The parvovirus NS 1 variant protein according to claim 3, wherein the NS 1 variants has a mutation at sites S283A (SEQ ID NO. 4), T363A (SEQ ID NO. 6), T394A (SEQ ID NO. 8), and T463A (SEQ ID NO. 10).
15. A DNA, coding for the parvovirus NS1 variant protein according to claim 4.
16. An expression vector, comprising the DNA according to claim 5.
17. A method of producing the parvovirus NS 1 variant protein according to claim 4, comprising the culturing of the transformant according to claim 8 under suitable conditions.
18. An antibody, directed against the parvovirus NS 1 variant protein according to claim 4.

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Parvovirus NS1 Variants

The present invention relates to parvovirus NS1 variants, DNAs coding for them and methods of producing the parvovirus NS1 variants. Furthermore, this invention concerns antibodies directed against the parvovirus NS1 variants as well as the use of the DNAs and the parvovirus NS1 variants.

Parvovirus designates a genus of the virus family Parvoviridae. The parvovirus genus comprises a number of small, icosaedric viruses that can replicate in the absence of a helper virus. Parvovirus contains a single-stranded DNA having a length of about 5.000 bp. At the 3' and 5' ends of the DNA there is one palindromic sequence each. The DNA codes for two capsid proteins, VP1 and VP2, as well as for two regulatory non-structure proteins, NS-1 and NS-2. The latter proteins are phosphorylated and show nuclear or both cytoplasmic and nuclear localization, respectively. NS1 is necessary for viral DNA replication and participates in the regulation of viral gene expression. Particularly, NS1 transactivates the promoter P38 and exhibits DNA-binding, helicase and DNA-nicking activities. Furthermore, NS1 induces cytotoxic and/or cytostatic stress in sensitive host cells.

Parvoviruses are usually well-tolerated by populations of their natural host, in which they persist without apparent pathological signs. This is due to both the protection of foetuses and neonates by maternal immunity, and the striking restriction of parvovirus replication to a narrow range of target proliferating tissues in adult animals. This host tolerance concerns especially rodent parvoviruses, for example the minute virus of mice (MVM) and H-1 virus in their respective natural hosts, namely mice and rats. In addition, humans can be infected with the latter viruses, without any evidence of

associated deleterious effects from existing epidemiological studies and clinical trials. On the other hand, it is known that certain parvoviruses, and especially rodent parvoviruses, are both oncotropic, i.e. accumulate preferentially in neoplastic versus normal tissues, and oncosuppressive, i.e. have a tumor-suppressive effect towards tumor cells, in various animal models. At least part of the oncosuppressive effect is thought to be due to a direct oncolytic action mediated by NS1. This oncosuppressive effect was also demonstrated against human tumor cells transplanted in recipient animals.

It is considered to use parvoviruses for therapeutic purposes. On the one hand, it seems to be of interest to use parvoviruses as vectors for therapeutic genes, i.e. for introducing such genes into the genome of cells. On the other hand, it is considered to use NS1 of parvoviruses as a toxin for treating tumoral diseases. However, initial experiments showed unsatisfactory results.

Therefore, it is the object of the present invention to provide a product by which parvoviruses and NS1 thereof, respectively, can be used for the above purposes.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on the applicant's findings that it is possible to interfere with the activities of parvovirus NS1 so as to shift the equilibrium existing between the DNA replication and transcription activities (a) and the cytotoxicity activity (b). In particular, he produced parvovirus NS1 variants in which the DNA replication and transcription activities (a) are reduced and eliminated, respectively, whereas the cytotoxicity activity (b) is maintained or raised. Moreover, he produced parvovirus NS1 variants in which the cytotoxicity activity (b) is reduced and eliminated, respectively, whereas the DNA replication and transcription activities (a) are

maintained or raised. Examples of such parvovirus NS1 variants are indicated in Table 1 and figure 1. In addition, the applicant recognized that the above parvovirus NS1 variants and expression vectors coding for them, particularly parvoviruses, respectively, are suitable for therapeutic purposes.

According to the invention, the applicant's findings are used to provide a parvovirus NS1 variant in which the equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b) is shifted.

The expression "parvovirus" comprises any parvovirus, particularly a rodent parvovirus, such as minute virus of mice (MVM) and H-1 virus.

The expression "the equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b) is shifted" refers to the fact that in a parvovirus NS1 variant according to the invention such an equilibrium is shifted as compared to the parvovirus NS1 wild-type. In particular, the equilibrium can be shifted to the effect that the DNA replication and transcription activities (a) are reduced and eliminated, respectively, whereas the cytotoxicity activity (b) is maintained or raised. The cytotoxicity activity (b) can also be reduced and eliminated, respectively, whereas the DNA replication and transcription activities (a) are maintained or raised. Such an equilibrium can be determined by various methods. As regards the determination of the DNA replication activity, reference is made e.g. to methods described in Legendre and Rommelaere, 1992, J. Virol. 66, 5705; Cotmore et al., 1992, Virology 190, 365; Cotmore et al., 1993, J. Virol. 67, 1579; Cotmore and Tattersall, 1994, Embo. J. 13, 4145. As to the determination of the transcription activity reference is made to methods described e.g. in Rhode and Richards, 1987, J. Virol. 61, 2807. Regarding the determination of the cytotoxicity activity reference is made to the below examples.

According to the invention parvovirus NS1 variants are preferred in which the shift of equilibrium is achieved by mutation of one or several phosphorylation sites. Particularly preferred are parvovirus NS1 variants which have a mutation at one or several of the phosphorylation sites 283, 363, 394 and 463. Even more preferred are the parvovirus NS1 variants S283A, T363A, T394A and T463A, which are indicated in Table 1 and figure 1. In S283A, a serine is exchanged by an alanine at position 283, in T363A a threonine is exchanged by alanine at position 363, in T394A a threonine is exchanged by alanine at position 394 and in T 463A a threonine is exchanged by alanine at position 463.

A further subject matter of the present invention relates to a nucleic acid, particularly a DNA, which codes for an above parvovirus NS1 variant. Such a DNA comprises preferably:

- (a) the DNA of fig. 1.1, 1.2, 1.3 and 1.4, respectively
- (b) a DNA hybridizing with the DNA from (a), said DNA comprising the mutated phosphorylation site of the DNA from (a), or
- (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.

The DNA of (a) was deposited with DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkulturen) on Aug. 11, 1999, i.e. fig. 1.1 as *Escherichia coli* pRSV-NS:S283A under DSM 12994, fig. 1.2 as *Escherichia coli* pRSV-NS:T363A under DSM 12995, fig. 1.3. as *Escherichia coli* pRSV-NS:T394A under DSM 12996 and fig. 1.4 as *Escherichia coli* pRSV-NS:T463A under DSM 12997.

The expression "hybridizing DNA" refers to a DNA which hybridizes with a DNA from (a) under normal conditions, particularly at 20(C below the melting point of the DNA. In this connection, the expression "hybridizing" refers to conventional hybridization conditions, preferably to hybridization conditions where 5xSSPE, 1 % SDS, 1xDenhardt's solution are used as solution and the hybridization temperatures are between 35(C and

70°C, preferably 65°C. The hybridization is followed by a wash step first carried out with 2xSSC, 1 % SDS and then with 0.2xSSC at temperatures between 35°C and 70°C, preferably at 65°C. Furthermore, reference is made to Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY (1989).

A DNA according to the invention can be present in a vector and expression vector, respectively. A person skilled in the art is familiar with examples thereof. In the case of an expression vector for E. coli these are e.g. pGEMEX, pUC derivatives, pGEX-2T, pET3b, T7 based expression vectors and pQE-8. For the expression in yeast, e.g. pY100 and Ycpad1 have to be mentioned while e.g. pKCR, pEFBOS, cDM8, pMSCND, and pCEV4 have to be indicated for the expression in animal cells. The baculovirus expression vector pAcSGHisNT-A is especially suitable for the expression in insect cells.

In a preferred embodiment, the vector containing the DNA according to the invention is a virus, e.g. an adenovirus, vaccinia virus, an AAV virus or a parvovirus, such as MVM or H-1, a parvovirus being preferred. The vector may also be a retrovirus, such as MoMuLV, MoMuLV, HaMuSV, MuMTV, RSV or GaLV.

For constructing expression vectors which contain the DNA according to the invention, it is possible to use general methods known in the art. These methods include e.g. in vitro recombination techniques, synthetic methods and in vivo recombination methods as described in Sambrook et al., supra, for example.

Furthermore, the present invention relates to host cells which contain the above described vectors. These host cells include bacteria, yeast, insect and animal cells, preferably mammalian cells. The E. coli strains HB101, DH1, x1776, JM101, JM109, BL21, XL1Blue and SG 13009, the yeast strain Saccharomyces cerevisiae and the animal cells L, A9, 3T3, FM3A, CHO, COS,

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particular, parvovirus NS1 variants are provided which have a reduced or no cytotoxicity activity, whereas the DNA replication and transcription activities are maintained or increased. Parvovirus NS1 variants are also provided in which the DNA replication and transcription activities are reduced and eliminated, respectively, whereas the cytotoxicity activity is maintained or raised. Thus, the present invention provides products which are suitable for therapeutic purposes. In particular, expression vectors according to the invention, e.g. parvoviruses, can be used for gene-therapeutic measures. Moreover, parvovirus NS1 variants according to the invention are suitable as toxins, e.g. for treating tumoral diseases.

Therefore, a kit is also provided for the application of the present invention. This kit comprises the following:

- (a) a parvovirus NS1 variant according to the invention,
- (b) a DNA according to the invention, e.g. an expression vector, particularly a parvovirus,
- (c) an antibody according to the invention, as well as
- (d) conventional auxiliary agents, such as solvents, buffers, carriers, markers and controls.

Of component (a) to (d) one or more representatives can be present each.

#### Brief description of the drawings

Fig. 1 shows the DNA and amino acid sequences of parvovirus NS1 variants according to the invention (fig. 1.1, 1.2, 1.3 and 1.4) as compared to a parvovirus NS1 wild-type. In this connection, the mutated sites in the parvovirus NS1 variants according to the invention are labeled each.

The present invention is explained by the examples.



**Example 1: Preparation and purification of NS1 variants according to the invention**

The DNA of the NS1 variant S283A according to the invention was provided as an EcoRV to BstEII fragment obtained by chimeric PCR using two mutagenic primers. This fragment was then inserted into the corresponding cleaved expression vector pTHisNS1 (Nuesch et al., Virology 209, (1995), 122) to obtain pTHisNS1:S283A. Such a vector codes for a fusion protein comprising 6 histidine residues (N terminus partner) and S283A of Fig. 1 (C terminus partner). For expression and purification of S283A the NS1 gene under control of the bacteriophage T7 promoter was transferred into vaccinia virus and expressed in eucaryotic cells by double infection together with vTF7-3 (a vaccinia virus expressing the bacteriophage T7 DNA polymerase). 18 hrs post infection cells were harvested and nuclear extracts prepared. The histidine tagged S283A was then purified by affinity chromatography on Ni-NTA agarose and analyzed by 10 % SDS-PAGE (Nuesch et al., supra).

It showed that a parvovirus NS1 variant according to the invention can be prepared in highly pure form.

The NS1 variants T363A, T394A, and T463A were also produced and purified in the same way.

**Example 2: Preparation and detection of an antibody according to the invention**

Tubes were coated with purified NS1 variants prepared as in example 1 and monoclonal antibodies (e.g. scFv) specifically binding to S283A were isolated from human synthetic VH+VL scFv phage library (Griffith et al., EMBO J., 13, (1994), 3245) according to standard panning protocols after >5 isolation and amplification procedures. The variable region of the isolated scFv harbored in the phagemid were sequenced to identify NS1

variant interacting partner proteins harboring such binding motifs from comparison with known genes in the gene bank.

It showed that monoclonal antibodies according to the invention can be isolated.

In addition, the NS1 variants were used for immunization of animals in order to obtain poly- or monoclonal antibodies.

**Example 3: Characterization of the parvovirus NS1 variants S283A, T363A, T394A and T463A according to the invention**

The characterization of the parvovirus NS1 variants comprised the determination of the DNA replication, transcription, cytotoxicity, DNA binding, nicking and helicase activities. Known methods were used for this purpose (cf. description, supra). As regards the determination of the helicase activity reference is made to Stahl et al. 1986, EMBO J. 5, 1999. As to the determination of the nicking activity reference is made to Christensen et al., 1997, J. Virol. 71, 1405 and Nuesch et al., 1995, supra. Regarding the determination of the DNA binding reference is made to Cotmore et al. 1995, J. Virol. 69, 1652. As far as the determination of the cytotoxicity activity is concerned, the following steps were carried out:

NS1 variants were transferred into an expression vector containing the NS1 gene under the control of the parvovirus MVMP4 promoter (genuine promoter driving the non-structural genes of MVM), and the green fluorescent protein (EGFP) under control of an additional promoter. These constructs were then transfected into A9 cells using lipofectamine (GibcoBRL) according to the manufacturer's instruction and the impact of the NS1 variant on the viability of the cells tested in time course experiments. Transfected cells were identified by fluorescence of the EGFP. Toxic effects were determined in comparison to wild type NS1 or a vector containing no NS1 gene

as a function of time as well as a measure of cytopathic changes on the cell morphology.

The data indicated in Table 1 were obtained:

Table 1

	S283A	T363A	T394A	T463A	wt
P38-TA	+	-	-	++++	++++
ACCA	+	++++	++	++	++
Nick-1	+	-	-	+++	+++
Nick-2	+++	-	-	++++	++++
Nick-3	++	-	-		++++
Heli	++	-	(+)	++++	++++
Rep	+	-	-	+	++++
Cyto	+++++	++	+++	(+)	+++

**Example 4: NS1 variants' expression after transduction using recombinant viral vectors**

NS1 expression cassettes containing the NS1 variants according to the invention under control of the parvoviral P4 promoter and a 3'untranslated region from parvovirus MVM to ensure stability and translation of the gene product, were transferred either in a parvovirus genome background as exemplified in example 3, or a heterologous viral genome background, such as vaccinia virus (example 1) or adenovirus. Promoter and terminator regions were exchanged according to the requirements. The nucleic acids containing the NS1 variants were then packaged either in vivo (after transient transfection into eucaryotic cells) or in vitro and the packaged transducing particles were isolated. These transducing units containing NS1 variants were used either for studies concerning gene regulation in tissue culture or animals, but also as therapeutic agents either alone or in combination with other agents (such as cytokines) in gene and cancer therapy

approaches.

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## Claims

1. A parvovirus NS1 variant having a shifted equilibrium between the DNA replication and transcription activities (a) and the cytotoxicity activity (b), wherein
  - 5 - the activities (a) are reduced and eliminated, respectively, and activity (b) is maintained or increased or
  - 10 - activity (b) is reduced and eliminated, respectively, and the activities (a) are maintained or increased.
2. The parvovirus NS1 variant according to claim 1, wherein one or several phosphorylation sites are mutated.
3. The parvovirus NS1 variant according to claim 2, wherein the mutations are located at sites 283, 363, 394 and/or 463.
4. The parvovirus NS1 variant according to claim 2 or 3, namely the NS1 variants S283A, T363A, T394A, and T463A.
5. A DNA, coding for the parvovirus NS1 variant according to any one of claims 1 to 4.
6. The DNA according to claim 5, wherein the DNA comprises:
  - (a) the DNA of figure 1,
  - (b) a DNA hybridizing with the DNA from (a), said DNA comprising the mutated phosphorylation site of the DNA from (a), or
  - (c) a DNA related to the DNA from (a) or (b) via the degenerated genetic code.
7. An expression vector, comprising the DNA according to

claim 5 or 6.

8. A transformant, containing the expression vector according to claim 7.

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9. A method of producing the parvovirus NS1 variant according to any one of claims 1 to 4, comprising the culturing of the transformant according to claim 8 under suitable conditions.

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10. An antibody, directed against the parvovirus NS1 variant according to any one of claims 1 to 4.

11. Kit comprising:

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- (a) a parvovirus NS1 variant according to the invention,
  - (b) a DNA according to the invention, e.g. an expression vector, particularly a parvovirus,
  - (c) an antibody according to the invention, as well as
  - (d) conventional auxiliary agents, such as solvents,
- 20 buffers, carriers, markers and controls,

wherein of components (a) to (d) one or more representatives can be present each.

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12. Use of the parvovirus NS1 variant according to any one of claims 1 to 4 as a toxin for treating tumoral diseases.

13. Use of the DNA according to claim 7 as a vector for gene therapy.

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(57) Abstract: The present invention relates to a parvovirus NS1 variant having a shifted equilibrium between the DNA replica-  
tion and transcription activities (a) and the cytotoxicity activity (b). Furthermore, this invention relates to DNAs coding for these  
parvovirus NS1 variants and methods of producing them. Additionally, this invention concerns antibodies directed against the par-  
vovirus NS1 variants as well as the use of the DNAs and the parvovirus NS1 variants.

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Wild-type NS1

	ATGGCTGGAAGTCTTA	CTTACTCTGATGAAGTTTTGGGAGACCAACAACTGGTTAAAGGAAAAA	
261	-+-----+	-+-----+	320
	TACCGACCTTTACGAATGAGACTACTTCAA	AACCCTCGTTGGETTGACCAATTCCTTTTT	
	M A G N A Y S D E V L G A T N W L K E K	-	
	AGTAACCAGGAAGTGT	TCTCATTTGTTTTTAAAAATGAAAATGTTCAACTGAATGCAAAA	
321	-+-----+	-+-----+	380
	TCATTGGTCCTTCACAAGAGTAAACAAAAA	TTTTACTTTTACAAGTTGACTTACCTTTT	
	S N Q E V F S F V F K N E N V Q L N G K	-	
	GATATCGGATGGAATAGTTACAAAAAAGAGCTGCAGGAGGACGAGCTGAAATC	TTTACAA	
381	-+-----+	-+-----+	440
	CTATAGCCTACCTTATCAATGTTTTTCTCGACGTCCTCCTGCTCGACTTTAGA	AATGTT	
	D I G W N S Y K K E L Q E D E L K S L Q	-	
	CGAGGAGCGGAAACTACTTGGGACCAAAGCGAGGACATGGAATGGGAAACCACAGTGGAT		
441	-+-----+	-+-----+	500
	GCTCCTCGCCTTTGATGAACCCTGGTTTCGCTCCTGTACCTTACCCTTTGGTGT	CACCTA	
	R G A E T T W D Q S E D M E W E T T V D	-	
	GAAATGACCAAAAAAGCAAGTATTCATTTTTGATTCTTTGGTTAAAAAATGTTTATTTGAA		
501	-+-----+	-+-----+	560
	CTTTACTGGTTTTTCGTTCCATAAGTAAAACTAAGAAACCAATTTTTTACAAATAAACTT		
	E M T K K Q V F I F D S L V K K C L F E	-	
	GTGCTTAACACAAAGAATATATTTCTCGGTGATGTTAATTGGTTTGTGCAACATGAATGG		
561	-+-----+	-+-----+	620
	CACGAATTGTGTTTCTTATATAAAGGACCACTACAATTAACCAACACGTTGTACTTACC		
	V L N T K N I F P G D V N W F V Q H E W	-	
	GGAAAAGACCAAGGCTGGCACTGCCATGTACTAATTGGAGGAAAGGACTTTAGTCAAGCT		
621	-+-----+	-+-----+	680
	CCTTTTCTGGTTCCGACCGTGACGGTACATGATTAACCTCCTTTCTGAAATCAGTTTCA		
	G K D Q G W H C H V L I G G K D F S Q A	-	
	CAAGGGAAATGGTGGACAAGGCAACTAAATGTTTACTGGAGCAGATGGTTGGTAACAGCC		
681	-+-----+	-+-----+	740
	GTTCCCTTTACCACCTCTTCCGTTGATTACAAATGACCTCGTCTACCAACCATTTGTCCG		
	Q G K W W R R Q L N V Y W S R W L V T A	-	
	TGTAATGTGCAACTAACACCAGCTGAAAGAATTAAACTAAGAGAAATAGCAGAAGACAAT		
741	-+-----+	-+-----+	800



Fig. 1 (Fortsetzung I)

ACATTACACGTTGATTGTGGTCGACTTTCTTAATTTGATTCTCTTTATCGTCTTCTGTIA  
C N V Q L T P A E R I K L R E I A E D N -  
GAGTGGGTTACTCTACTTACTTATAAGCATAAGCAAACCAAAAAGACTATAACCAAGTGT  
801 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 860  
CTCACCCAATGAGATGAATGAATATTCGTATTCGTTTGGTTTTTCTGATATGGTTCACA  
E W V T L L T Y K H K Q T K K D Y T K C -  
GTTCTTTTTTGGAAACATGATTGCTTACTATTTTTTAACTAAAAAGAAAATAAGCACTAGT  
861 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 920  
CAAGAAAAACCTTTGTACTAACGAATGATAAAAAATTGATTTTTCTTTTATTCGTGATCA  
V L F G N M I A Y Y F L T K K K I S T S -  
CCACCAAGAGACGGAGGCTATTTTTCTTAGCAGTGAAGTCTGGCTGGAAACTAACTTTTTTA  
921 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 980  
GGTGGTTCTCTGCCTCCGATAAAAGAATCGTCACTGAGACCGACCTTTTGATTGAAAAAT  
P P R D G G Y F L S S D S G W K T N F L -  
AAAGAAGGCGAGCGCCATCTAGTGAGCAACTATACACTGATGACATGCGGCCAGAAACG  
981 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1040  
TTTCTTCCGCTCGCGGTAGATCACTCGTTTGATATGTGACTACTGTACGCCGGTCTTTTC  
K E G E R H L V S K L Y T D D M R P E T -  
GTTGAAACCAACAGTAACCACTGCGCAGGAACTAAGCGCGGCAGAATTCAAACATAAAAA  
1041 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1100  
CAACTTTGGTGTTCATTGGTGACCGCTCCTTTGATTGCGCGCGTCTTAAGTTTGATTTTTT  
V E T T V T T A Q E T K R G R I Q T K K -  
GAAGTTTCTATTAAAACTACACTTAAAGAGCTGGTGCATAAAAGAGTAACCTCACCAGAG  
1101 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1160  
CTTCAAAGATAAATTTTGATGTGAATTTCTCGACCACGTATTTTCTCATTTGGAGTGGTCTC  
E V S I K T T L K E L V H K R V T S P E -  
GACTGGATGATGATGCAGCCAGACACTTACATTGAAATGATGGCTCAACCAGGTGGAGAA  
1161 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1220  
CTGACCTACTACTACGTGGTCTGTCAATGTAACCTTTACTACCGAGTTGGTCCACCTCTT  
D W M M M Q P D S Y I E M M A Q P G G E -  
AACCTGCTGAAAAATACGCTAGAGATTGTACACTAACTCTAGCCAGAACCAGCA  
1221 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1280  
TTGGACGACTTTTTATGCGATCTCTAAACATGTGATTGAGATCGGTCTTGGTTTTGTCGT  
N L L K N T L E I C T L T L A R T K T A -  
TTTGACTTAATTTTAGAAAAAGCTGAAACCAGCAAACTAACTTTTCACTGCCTGAC  
1281 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1340  
AAACTGAATTAATCTTTTTTCGACTTTGGTCGTTTGATTGGTTGAAAAGTGACGGACTG  
F D L I L E K A E T S K L T N F S L P D -  
ACAAGAACCTGCAGAAATTTTTGCTTTTCATGGCTGGAACATGTTAAAGTTTGCCATGCT  
1341 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1400  
TGTTCTTGGACGTCTTAAAAACGAAAAGTACCGACCTTGATACAATTTCAAACGGTACGA  
T R T C R I F A F H G W N Y V K V C H A -

Fig. 1 (Fortsetzung II)

ATTTGCTGTGTTTTAAACAGACAAGGAGGCAAAAGAAATACTGTTTTATTTTCATGGACCA  
1401 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1460  
TAAACGACACAAAATTTGTCTGTTCTCCGTTTTCTTTATGACAAAATAAAGTACCTGGT  
  
I C C V L N R Q G G K R N T V L F H G P -  
  
GCCAGCACAGGCAAATCTATTATTGCACAAGCCATAGCACAAGCAGTTGGCAATGTTGGT  
1461 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1520  
CGGTCGTGTCCGTTTAGATAATAACGTGTTCCGGTATCGTGTTCGTCAACCGTTACAACCA  
  
A S T G K S I I A Q A I A Q A V G N V G -  
  
TGCTATAATGCAGCCAATGTAACTTTCCATTTAATGACTGTACCAACAAGAACTTGATT  
1521 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1580  
ACGATATTACGTCCGTTTACATTTGAAAGGTAAATTACTGACATGGTTGTTCTTGAACATA  
  
C Y N A A N V N F P F N D C T N K N L I -  
  
TGGGTACAAGAAGCTGGTAACCTTTGGACAGCAAGTAAACCAGTTTAAAGCCATTTGCTCT  
1581 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1640  
ACCCATCTTCTTCGACCATTTGAAACCTGTCTTCATTTGGTCAAATTTCCGTTAAACGAGA  
  
W V E E A G N F G Q Q V N Q F K A I C S -  
  
GGTCAAACTATTTCGCATTGATCAAAAAGGAAAAGGCAGCAACAGATTGAACCAACACCA  
1641 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1700  
CCAGTTTGATAAGCGTAAGTACTAGTTTTCTTTTCCGTCGTTTGTCTAACTTGGTTGTGGT  
  
G Q T I R I D Q K G K G S K Q I E P T P -  
  
GTCATCATGACCACAAATGAGAACATTACAGTGGTCAGAAATAGGCTGCCAAGAAAGACCA  
1701 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1760  
CAGTACTACTGGTGTCTTACTCTTGTAAATGTCACCAGTCTTATCCGACGCTTCTTTCTGGT  
  
V I M T T N E N I T V V R I G C E E R P -  
  
GAACACACTCAACCAATCAGAGACAGAATGCTTAAACATTATCTAACACATACCTTGCCT  
1761 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1820  
CTTGTGTGAGTTGGTTAGTCTCTGTCTTACGAATTGTAAGTAGATTGTGTATGGAACGGA  
  
E H T Q P I R D R M L N I H L T H T L P -  
  
GGTGACTTTGGTTTGGTTGACAAAATGAATGGCCCATGATTTGTGCTTGGTTGGTAAAG  
1821 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1880  
CCACTGAAACCAACCAACTGTTTTTACTTACCGGTACTAAACACGAACCAACCACTTTC  
  
G D F G L V D K N E W P M I C A W L V K -  
  
AATGTTTACCAATCTACCATGGCAAGCTACTGTGCTAAATGGGGCAAAGTTCTTGATTGG  
1881 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 1940  
TTACCAATGGTTAGATGGTACCGTTCGATGACACGATTTACCCCGTTTCAAGGACTAACC  
  
N G Y Q S T M A S Y C A K W G K V P D W -  
  
TCAGAAAACCTGGGCGGAGCCAAAGGTGCCAACTCCTATAAATTTACTAGGTTCCGGCACGC  
1941 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2000  
AGTCTTTTGAACCCGCTCGGTTTCCACGGTTGAGGATATTTAAATGATCCAAGCCGTGCG  
  
S E N W A E P K V P T P I N L L G S A R -  
  
TCACCATTACGACACCCGAAAAGTACGCCCTCTCAGCCAGAACTATGCACTAACTCCACTT  
2001 -----+-----+-----+-----+-----+-----+-----+-----+-----+-----+ 2060  
AGTGGTAAGTGCTGTGGCTTTTCATGCGGAGAGTCCGCTCTGATACGTGATTGAGGTGAA

Fig. 1 (Fortsetzung III)

S P F T T P K. S T P L S Q N Y A L T P L -  
 GCATCGGATCTCGAGGACCTGGCTTTAGAGCCTTGGAGCACACCAAATACTCCTGTTGCG  
 2061 -----+----- 2120  
 CGTAGCCTAGAGCTCCTGGACCGAAATCTCGGAACCTCGTGCTGTTTATGAGGACAACGC  
 A S D L E D L A L E P W S T P N T P V A -  
 GGCACTGCAGAAACCCAGAACACTGGGGAAGCTGGTTCCAAAGCCTGCCAAGATGGTCAA  
 2121 -----+----- 2180  
 CCGTGACGTCCTTTGGGTCTTTGTGACCCCTTCGACCAAGGTTTCGGACGGTTCTACCACTT  
 G T A E T Q N T G E A G S K A C Q D G Q -  
 CTGAGCCCCAACTTGGTCAGAGATCGAGGAGGATTTGAGAGCGTGCTTCGGTGCGGAACCG  
 2181 -----+----- 2240  
 GACTCGGGTTGAACCAAGTCTCTAGCTCCTCCTAAACTCTCGCACGAAGCCACGCCTTGGC  
 L S P T W S E I E E D L R A C F G A E P -  
 TTGAAGAAAGACTTCAGCGAGCCGCTGAACTTGGACTAA  
 2241 -----+----- 2279  
 AACTTCTTTTCTGAAGTCGCTCGGGCGACTTGAACCTGATT  
 L K K D F S E P L N L D \* -

Fig. 1.1

1100 - 261 Wildtype-NS1-Sequence

GAAGTTTCTATTAAACTACACTTAAAGAGCTGGTGCATAAAAGAGTAACCTCACCAGAG  
1101 -----+-----+-----+-----+-----+-----+ 1160  
CTTCAAGGATAATTTTGATGTGAATTTCTCGACCACGTATTTTCTCATTGGAGTGGTCTC  
E V [S] I K T T L K E L V H K R V T S P E -  
→ A 5283A

1161 - 2279 Wildtype-NS1-Sequence

Fig. 1.2

1340 - 261 Wildtype-NS1-Sequence

ACAAGTACCTGCAGAAATTTTGCTTTTCATGGCTGGAAGTATGTTAAAGTTTGCCATGCT  
1341 -----+-----+-----+-----+-----+ 1400  
TGTTCCTGGACGTCTTAAAAACGAAAAGTACCGACCTTGATACAATTTCAAACGGTACGA  
T R [T] C R I F A F H G W N Y V K V C H A -  
→ A T363A

1401 - 2279 Wildtype-NS1-Sequence

1400 - 261 Wildtype-NS1-Sequence

1461 - 2279 Wildtype-NS1-Sequence

1640 - 261 Wildtype-NS1-Sequence

1701 - 2279 Wildtype-NS1-Sequence

K 3039

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## PATENT APPLICATION

DECLARATION AND POWER OF ATTORNEY  
FOR PATENT APPLICATION

ATTORNEY DOCKET NO. 4121-136

As a below named inventor, I hereby declare that:

My residence/post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PARVOVIRUS NS 1 VARIANTS

the specification of which is attached hereto unless the following box is checked:

(X) was filed February 11, 2002 as US Application Serial No. 10/069,056 or PCT International Application

Number \_\_\_\_\_ and was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understood the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose all information which is material to patentability as defined in 37 CFR 1.56.

## Foreign Application(s) and/or Claim of Foreign Priority

I hereby claim foreign priority benefits under Title 35, United States Code Section 119(a-d) or 365(b) of any foreign application(s) for patent or inventor(s) certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor(s) certificate having a filing date before that of the application on which priority is claimed:

COUNTRY	APPLICATION NUMBER	DATE FILED	PRIORITY CLAIMED UNDER 35 U.S.C. 119
EPO	99 115 161.4	13 August 1999	YES: <u>X</u> NO: _____
PCT	PCT/EP00/07835	11 August 2000	YES: <u>X</u> NO: _____

## U.S. Priority Claim

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NUMBER	FILING DATE	STATUS(patented/pending/abandoned)

## POWER OF ATTORNEY:

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) listed below to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Steven J. Hultquist, Reg. No. 28021Marianne Fuierer, Reg. No. 39983

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(919) 419-9350

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's Signature

Date

10069056 072902

DECLARATION AND POWER OF ATTORNEY  
FOR PATENT APPLICATION (continued)

ATTORNEY DOCKET NO. 4121-136

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Date

10.7.02